FOOD MICROORGANISMS: BUOYANT DENSITY CENTRIFUGATION

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Introduction

Density gradient centrifugation is an established technique for the separation and purification of eukaryotic and prokaryotic cells, viruses and subcellular components such as plasmids, mitochondria and nucleic acids. Using this technique, components may be separated based on their differences in density or size during centrifugation in a gradient medium. Gradient media that have been used include caesium chloride, sodium metrizoate, sucrose, Ficoll, Ludox®, Percoll® and BactXtractor™. One of the limitations with this technique has often been the properties of the gradient medium used. For instance, it is crucial that the gradient medium is at physiological ionic strength to avoid cell lysis or dehydration effects. Further, the gradient medium should be nontoxic and not affect the viability of the cells.

Density gradient centrifugation using different gradient media has been used to separate a wide range of microorganisms from various types of samples. For example, bacteria have been recovered from soil using sucrose gradients and different types of protozoa have been separated from river water, faeces, etc., using various gradient media. Further, gradient centrifugation in Percoll® has been used to distinguish subpopulations of pathogenic bacteria and to separate live from dead eukaryotic cells.

To some extent this technique has also been used to separate microorganisms from food, e.g. in the separation of bacteria from milk and natural yoghurt. The main benefits of using density centrifugation is its simplicity and speed in separating and concentrating intact organisms from foods while at the same time removing compounds that might interfere with or inhibit the detection method. The ability to remove inhibiting or interfering material present in food has been evaluated by subsequent detection of prepared microorganisms through methods of varying sensitivity such as traditional plate count procedures, the polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA), and ATP measurements.

The present work describes how to use buoyant density centrifugation and gives an example of how to design a procedure for the separation of microorganisms from a specific food. This approach has been successful for several food/microorganism combinations and it has been possible to separate and concentrate bacteria from food and to remove inhibitors sufficiently to allow detection of bacteria by both PCR and NASBA. However, in some cases, especially when the food contains denser components, there can be limitations which may be overcome by use of a two-layer technique also described here. The protocols presented are based on work described in Lindqvist *et al.* (1997), Lindqvist (1997) and Anonymous (1995) (see Further Reading).

General Theory and Methodology

Principles of Centrifugation

Equation [1] describes the sedimentation of a sphere in a centrifugal field:

$$v = [d^2(\rho - \rho_1)/18\eta] \times g \qquad [1]$$

where v = sedimentation rate, d = diameter of the particle (hydrodynamically equivalent sphere), ρ = particle density, ρ_1 = liquid density, η = viscosity of the medium and g = centrifugal force.

From this relationship it can be seen that the sedimentation rate of a particle is proportional to its size and to the density difference between the particle and the medium. Also, the sedimentation rate is zero when the density of the particle is equal to the density of the surrounding medium. Further, the sedimentation rate decreases with increasing viscosity of the medium and increases with increasing centrifugal force applied. From this it also follows that separation depending on the conditions chosen may be carried out based on either the size (rate zonal centrifugation) or the density differences (isopycnic centrifugation) between particles. In the latter case, each particle will sediment to an equilibrium position in the gradient where the gradient density is equal to the density of the particle. In the present work, the isopycnic technique is discussed.

The specific cell density, i.e. cell weight/cell volume when measured by buoyancy in a given medium capable of forming density gradients, is referred to as the buoyant density. Consequently, anything that affects the size of the microorganism, e.g. osmotic

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conditions and growth phase, may also affect their buoyant densities and, thus, separation. This stresses the importance of the properties of the gradient medium and of using standardized conditions when developing and using the separation protocol.

Gradient Media

Two gradient media with favourable properties for work with microorganisms are Percoll® and BactXtractor™. The composition and properties of these gradient media are similar (see below). The most important difference is that BactXtractor may be autoclaved after NaCl and peptone have been added to prepare a standard isotonic medium (SIM). Percoll and BactXtractor are nontoxic, have a low osmotic pressure and viscosity. These media consist of colloidal silica particles of 15–30 nm diameter coated with polyvinylpyrrolidone (PVP). They can form self-generated gradients in the range of 1.0–1.3 g mL^{-1}, which correspond to the cell densities of many microorganisms. When a solution of Percoll® (or BactXtractor™) is centrifuged at $> 10\,000 \times g$ in an angle-head rotor, the coated and hydrated silica particles will sediment resulting in an uneven distribution of particles and the formation of a self-generated density gradient. The gradient is formed isometrically around the initial density of the gradient medium and becomes steeper with centrifugation time. The shape of the gradient can be visualized by the use of coloured density marker beads and is related approximately linearly to the g force and time of the centrifugation. Rotor geometry and the size of the tubes also have a marked effect on gradient shape. In contrast to the self-generated continuous gradient, for some applications a uniform density centrifugation using one (cushion) or several layers (step gradient) of the gradient medium is preferred. The latter approaches are discussed here for the separation of bacteria from food.

Overview of Methodology

When designing a separation protocol, the same procedure may be followed independent of the subsequent detection method. This procedure includes the following steps (**Figure 1**): (1) determination of the buoyant density of the microorganism; (2) determination of the buoyant density of the food; (3) selection of the concentration of the gradient medium to be used in the separation of the microorganism from food; (4) evaluation of the separation protocol with the desired detection method; and (5) optimization of the protocol if necessary.

Preparation of the Gradient Medium

The gradient media, Percoll® (Pharmacia Biotech, Sweden) or BactXtractor™ (QRAB, Uppsala, Sweden) have a density of around 1.130 g mL^{-1}. Before use, the medium is made isotonic with physiological saline by aseptically adding 8.5 g L^{-1} NaCl, and

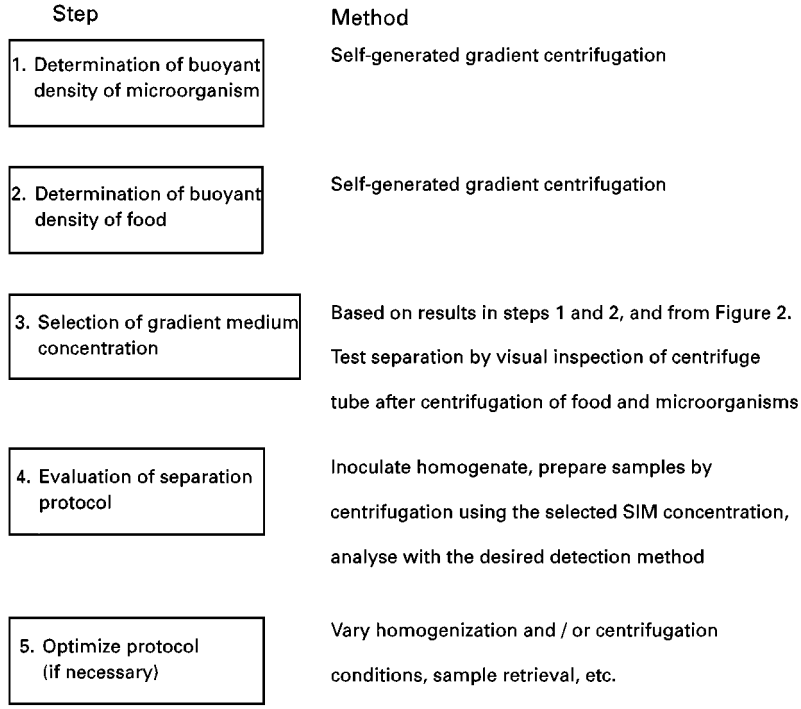
Step
Method
1. Determination of buoyant density of microorganism
Self-generated gradient centrifugation
2. Determination of buoyant density of food
Self-generated gradient centrifugation
3. Selection of gradient medium concentration
Based on results in steps 1 and 2, and from Figure 2.
Test separation by visual inspection of centrifuge tube after centrifugation of food and microorganisms
4. Evaluation of separation protocol
Inoculate homogenate, prepare samples by centrifugation using the selected SIM concentration, analyse with the desired detection method
5. Optimize protocol (if necessary)
Vary homogenization and / or centrifugation conditions, sample retrieval, etc.

Figure 1 Schematic overview of the sequential steps involved in designing a protocol for separation of microorganisms from food.

its suitability for maintaining microorganisms is improved by addition of 1.0 g L^{-1} peptone. This stock solution is termed 100% standard isotonic medium (100% SIM) and may be autoclaved if prepared with BactXtractor™. The solution of 100% SIM is diluted to the required concentration with the appropriate volume of peptone-water (8.5 g NaCl and 1.0 g peptone in 1 litre of Millipore water).

The density of the 100% SIM solution described above is calculated by the following formula:

$$\rho_s = [V_g \times \rho_g + m_{NaCl} + m_p]/V_g$$
$$= [100 \times 1.130 + 0.85 + 0.1]/100 = 1.1395 \quad [2]$$

where ρ_s = the density of 100% SIM (g mL^{-1}), V_g = the volume of gradient medium to be prepared (mL), assumed here to be 100 mL (the volume change by addition of solutes is negligible), ρ_g = the actual density of the gradient medium, assumed here to be 1.130 g mL^{-1}, m_{NaCl} = the amount of NaCl added (g), assumed here to be 0.85 g and m_p = the amount of peptone added (g), assumed here to be 0.1 g.

By using eqn [2], the density of 100% SIM was calculated to be 1.1395 g mL^{-1}, assuming that 100 mL of gradient medium with a density of 1.130 g mL^{-1} was mixed with NaCl and peptone. The relationship between density and concentration of SIM can be determined by plotting the calculated density of a 100% solution and the density of a 0% solution, i.e. the density of the diluent, as a function of percent SIM concentration and then determining the equation for the straight line between these two points:

$$\rho_{sy} = [(\rho_g - \rho_p)/(100)] \times C_s + \rho_p$$
$$= [(1.1395 - 1.0095)/100]C_s + 1.0095$$
$$= 0.0013 \times C_s + 1.0095 \quad [3]$$

where ρ_{sy} = the density of SIM of concentration C_s (g mL^{-1}), ρ_p = density of peptone water, estimated here to be 1.0095 g mL^{-1}, C_s = concentration of SIM (%) and ρ_g = the actual density of the gradient medium, assumed here to be 1.130 g mL^{-1}.

The line described by eqn [3] and shown in **Figure 2** is valid only for the properties of the gradient medium and diluent assumed in this work but similar graphs can easily be constructed for other experimental conditions. The graph can then be used to determine what concentration of SIM is required to produce a gradient medium of a particular density.

Determination of the Buoyant Densities of Microorganisms

Since the buoyant densities of microorganisms may be affected by a number of factors, it is important that they are handled and separated from food under standardized conditions. For instance, the buoyant densities of bacteria in Percoll® gradients have been shown to vary with growth rate and during the cell cycle for some bacteria but not for others. Further, efforts to inactivate bacteria by, for instance, boiling, heating, and low pH treatment have shown that,

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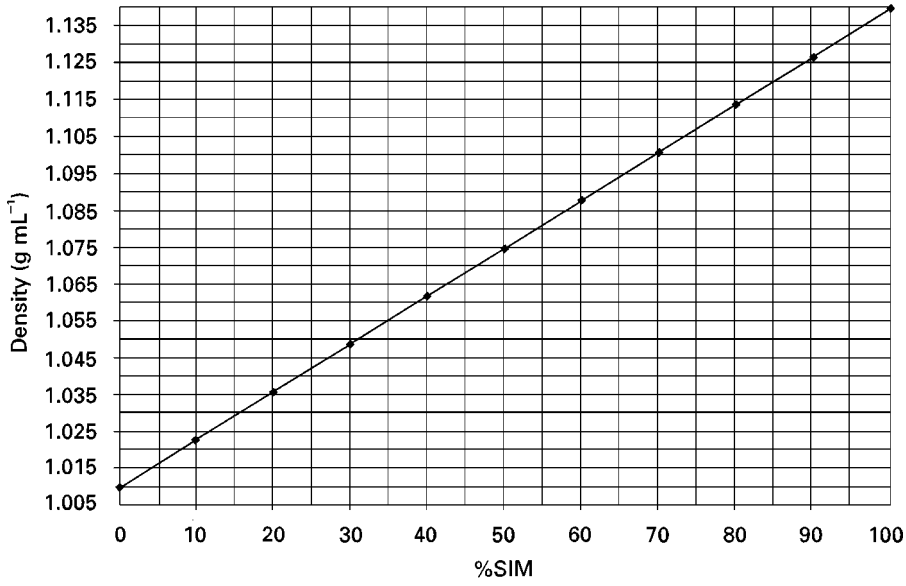

Figure 2 The relationship between density and concentration of the standard isotonic medium (SIM). The relationship described by this line is only an example and was calculated based on the conditions described in this work. The exact relationship must be calculated based on the density of the gradient medium and the diluent used.

depending on the treatment and the strain used, this may or may not affect their buoyant densities. Some variation in buoyant densities of a given strain may be expected depending on the culture media used, storage time after growth, etc., but ideally this is in a density range where separation is not affected. However, the presence of different subpopulations in the sample, e.g. log-phase and stationary cells, may result in a wide continuous distribution, or even separate bands, of microorganisms within the centrifuge tube.

Preparation of Microorganisms

A sufficient number of strains are cultured and treated under relevant conditions to collect data on the buoyant density of the microorganism and its variation. The number of cells loaded on to the gradient must be large enough to form a visible band in the centrifuge tube after centrifugation. In most cases a solution containing 10^8–10^9 cfu mL^{-1} is sufficient. The solution is prepared by centrifuging an appropriate volume of culture, washing, and resuspending in physiological saline or peptone-water. The washing and resuspension of cells in fresh physiological saline solution serves to suppress variations in osmotic pressure introduced by the presence of metabolites, etc.

Sampling Loading

The appropriate volume of the microorganism suspension or solution containing the density marker beads is carefully layered on the standard isotonic medium. There are no definite rules on how much sample it is possible to load on to the gradient and this must be tested empirically. The best SIM concentration to use depends of course on the microorganisms. In our work, concentrations between 50% and 80% and between 40% and 70% have been used with microorganisms and food, respectively. However, in addition to buoyant density, specific requirements on the amount of sample and the shape of the gradient may influence the scale of the experiment, i.e. rotor geometry and size of the tubes. **Figure 3** shows examples of a large scale and a small scale protocol.

Generation and Reading of the Gradient

The gradient is generated by centrifugation and is visualized by substituting the sample volume with a solution of colour-coded density marker beads (Pharmacia Biotech, Sweden) on top of the gradient medium. A volume of marker solution equivalent to the sample volume is prepared by adding approximately 5–10 μL of each marker bead to a physiological saline solution. During centrifugation, the density beads equilibrate at positions in the gradient corresponding to their densities. The distance of the differently coloured density beads from the bottom of the tube is recorded and the densities of the beads are plotted versus the position to generate a calibration curve (**Figure 4**). Similarly, the position of the microorganisms in the centrifuge tube is recorded and the corresponding buoyant density is read from the calibration curves. The best resolution is obtained in the steep part of the curves where a large distance in the centrifuge tubes corresponds to a small difference in density (Figure 4). By comparing the shapes and locations of the three gradients in **Figure 4** it can be seen that the resolution in the 60% SIM gradient is

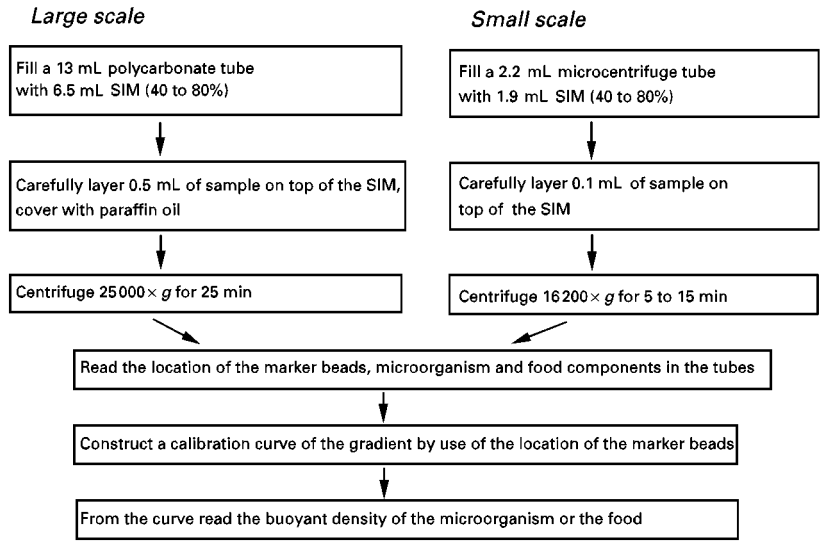
Large scale
Fill a 13 mL polycarbonate tube with 6.5 mL SIM (40 to 80%)
Carefully layer 0.5 mL of sample on top of the SIM, cover with paraffin oil
Centrifuge 25000× g for 25 min
Small scale
Fill a 2.2 mL microcentrifuge tube with 1.9 mL SIM (40 to 80%)
Carefully layer 0.1 mL of sample on top of the SIM
Centrifuge 16200× g for 5 to 15 min
Read the location of the marker beads, microorganism and food components in the tubes
Construct a calibration curve of the gradient by use of the location of the marker beads
From the curve read the buoyant density of the microorganism or the food

Figure 3 Example of two protocols for the determination of the buoyant density of microorganisms and food.

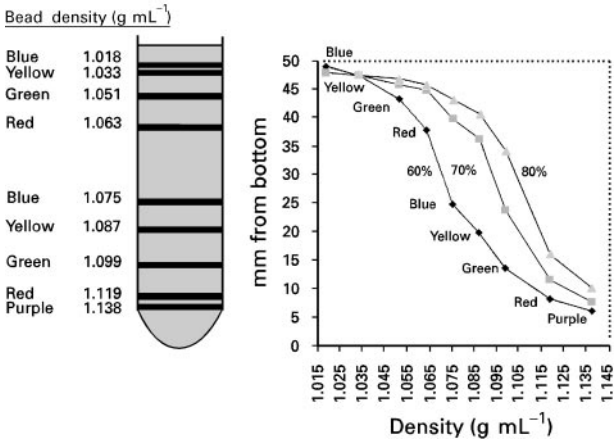

Figure 4 Illustration of how to construct calibration curves for the determination of the buoyant densities of microorganisms and food. The gradients were generated by layering 0.5 mL of density marker beads on 6.5 mL of 60%, 70% or 80% SIM and centrifuging at 25 000 × *g* for 25 min. Left: the positions of the different bands of colour-coded density marker beads in 60% SIM. Right: the curves were constructed by plotting the position and the density of the density marker beads in the diagram. The best resolution is obtained in the steep part of the curves, i.e. large difference in position corresponds to a small difference in density.

better at lower densities and vice versa for the 80% SIM. However, in addition to the selection of the SIM concentration, it is sometimes useful to vary the centrifugation time and speed when optimizing the gradient for a specific microorganism or food. In a given gradient, the width of the more or less well-defined band of microorganisms following centrifugation depends on the number of microorganisms and the range of buoyant densities present in the population under study.

Determination of the Buoyant Densities of Food

The food homogenate to be loaded on the gradient medium must be sufficiently concentrated to be visible in the centrifuge tube. Thus, a more concentrated homogenate is often used for this step than will be used in the final separation protocol. Typically a 1 : 1 to 1 : 10 (w/v) homogenate is suitable. The determination of the buoyant density of food is then carried out by centrifugation in a self-generated density gradient as described above for the microorganism.

Selecting the Concentration of the Gradient Medium

The simplest technique to separate microorganisms from food is to centrifuge the sample on a single layer of gradient medium of a uniform density (cushion centrifugation). During the previous steps the buoyant densities of the microorganism and of the food have been determined under the relevant conditions which will indicate if separation is possible, i.e. if there is a difference in buoyant densities that may be exploited. The food is generally less dense than the microorganisms and the density of the gradient medium is chosen so that it lies between the densities of the food and the microorganism. Thus, the microorganisms will be found in the bottom of the tube after centrifugation. The optimum concentration of SIM to use to obtain the cutoff density between food and microorganisms can be determined from the relationship between density and SIM concentration described in eqn [3] and **Figure 2**.

Separation by a Uniform Density Centrifugation

A quick way to test if the correct concentration of SIM has been selected is to run a uniform density centrifugation (see below) of a cell suspension and food homogenate, respectively. After centrifugation, food should remain on top of the gradient medium and cells should be visible in the lower part of the tube. Based on the exact position of microorganisms, the volume of sample that needs to be retrieved is determined. The sample volume is the amount of medium contained in the centrifuge tube from the upper band limit to the bottom of the tube.

In order to run a uniform density centrifugation, gradient formation during centrifugation should be avoided. This may be achieved either by centrifugation in a swing-out rotor or, if using an angle-head rotor, by centrifuging under conditions where gradient formation is negligible (e.g. low *g* forces or short centrifugation times).

Evaluation of Protocol

To evaluate the separation procedure, inoculated food homogenates are analysed with the desired method of detection, e.g. plate counts or PCR. The size of the centrifuge tubes to use depends on the amount of sample needed for detection as well as the number of samples to be analysed in a given time.

In **Figure 5**, examples of a large scale (larger sample capacity) and a small scale (larger sample throughput) protocol for separation of microorganisms from food are offered to assist in the selection of which centrifugation conditions to be used. Similar, or identical, protocols have been used to separate microorganisms, e.g. *Shigella* spp., *E. coli* O157:H7, *Yersinia enterocolitica*, *Campylobacter* spp. and

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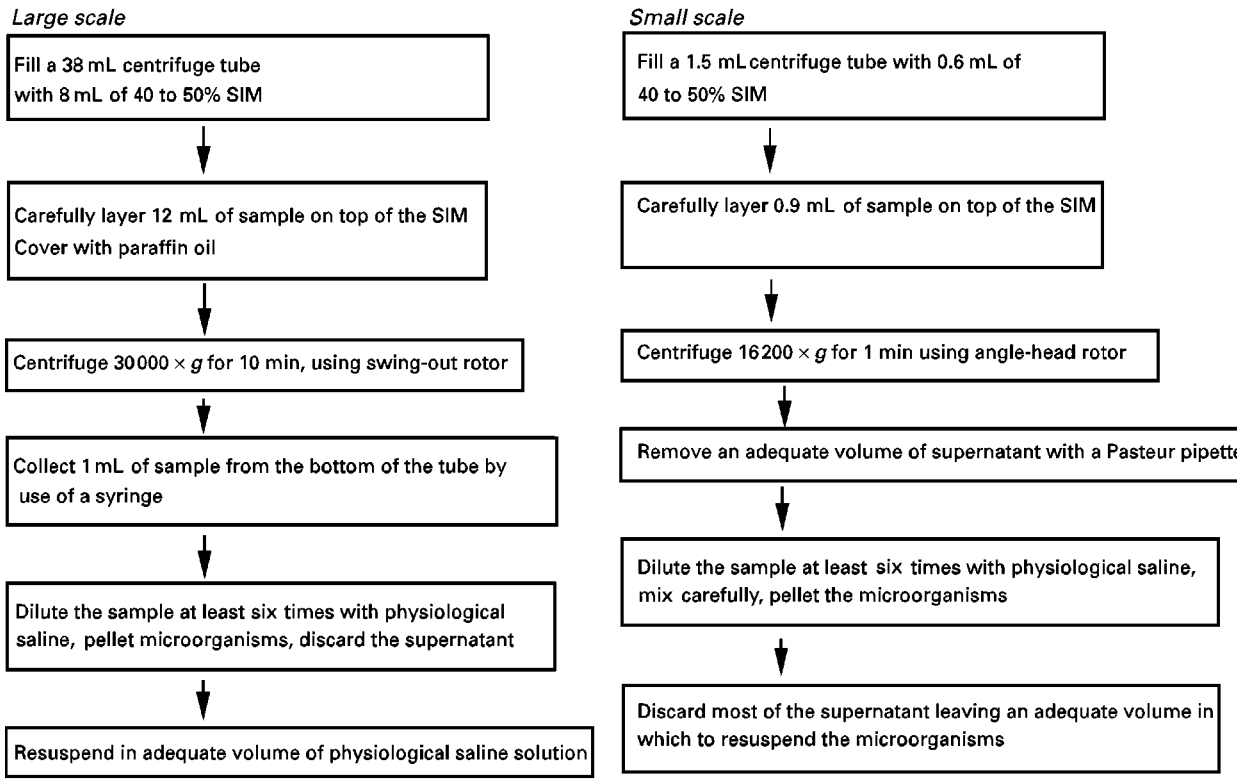
Large scale
Fill a 38 mL centrifuge tube with 8 mL of 40 to 50% SIM
Carefully layer 12 mL of sample on top of the SIM Cover with paraffin oil
Centrifuge 30000 × g for 10 min, using swing-out rotor
Collect 1 mL of sample from the bottom of the tube by use of a syringe
Dilute the sample at least six times with physiological saline, pellet microorganisms, discard the supernatant
Resuspend in adequate volume of physiological saline solution
Small scale
Fill a 1.5 mL centrifuge tube with 0.6 mL of 40 to 50% SIM
Carefully layer 0.9 mL of sample on top of the SIM
Centrifuge 16200 × g for 1 min using angle-head rotor
Remove an adequate volume of supernatant with a Pasteur pipette
Dilute the sample at least six times with physiological saline, mix carefully, pellet the microorganisms
Discard most of the supernatant leaving an adequate volume in which to resuspend the microorganisms

Figure 5 Example of two protocols for the separation of microorganisms from food by a uniform density centrifugation.

Zygosaccharomyces rouxii from different foods, e.g. raw beef, ground beef, different vegetables, shrimps, chicken, blackcurrant syrup, and milk, prior to detection by methods such as PCR, NASBA and plate count procedures.

Optimization of Protocol

If, due to incomplete separation, the detection limit is not satisfactory there are some key separation parameters that can be changed. Initially it can be helpful to add density marker beads corresponding to densities similar to those of the microorganisms during the uniform density centrifugation. The location of the beads will indicate where in the tube the microorganisms will be found and if the optimal SIM concentration has been used. The beads may also indicate if a gradient has formed during centrifugation.

Instead of sampling from above, as suggested in **Figure 5**, microorganisms can be retrieved by insertion of a syringe through the bottom of the centrifuge tube to avoid the mixing of inhibitory compounds or particles from the supernatant during cell removal.

If particles from the food end up in the treated sample, one can try to decrease the particle content in the sample volume prior to centrifugation by diluting the homogenate and/or preparing the homogenate in a stomacher bag with a filter bag. If this does not help, or if the food contains particles denser than the microorganism, it is possible to use two layers of gradient medium of different but uniform concentrations (step gradient). The density of the second layer is chosen to lie between that of the microorganisms of interest and the denser components of the food. This technique has been used to separate pathogenic bacteria in a blue cheese from the lighter cheese particles and from denser fungal mycelia. The sample is retrieved at the interface between the gradient layers and this position can be identified by centrifugation of an identical tube where the sample is replaced with density marker beads.

Another possibility, if the exact buoyant density of the microorganism is known such as in a well-defined and constant experimental system, is to perform the separation step using a continuous gradient centrifugation.

Future Developments

Buoyant density centrifugation is a general method which recovers nonattached microorganisms over a specific buoyant density. This suggests two possible areas for development and improvement. The first

would be to increase the fraction of nonattached cells by optimization of the homogenization or stomaching process. The second area is the exciting possibility of developing a separation protocol specific for single types of microorganisms, or a systematic or metabolic group of microorganisms. This may be achieved by manipulating the buoyant density of an organism through a selective uptake of a specific compound. Further, since it is possible to perform the separation on a micro scale, it may be feasible to design automated systems for sample preparation and analysis with a high sample capacity.

See also: **II/Centrifugation:** Theory of Centrifugation.

Further Reading

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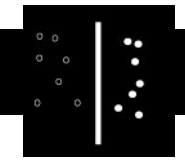

Membrane Separations

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One of the earliest successful industrial applications of membrane technology was in the food industry. In 1972, a dairy plant in New York began processing cheese whey by reverse osmosis. Membrane separations are now ubiquitous in the food industry, as shown in **Table 1**. The main use of reverse osmosis is the concentration of liquid foods, to complement or replace evaporation. Nanofiltration is used for desalting and de-acidification with partial concentration, while ultrafiltration is used for fractionation, concentration and purification of food streams. Microfiltration is used for clarification and removal of suspended matter to replace centrifuges and filter presses. It is also used for pasteurizing and sterilizing liquids instead of using heat. Electrodialysis is finding use for demineralization and de-acidification, as a possible partial replacement for ion exchange. To date, pervaporation applications are few in the food industry, although it could be used for purification of volatile aroma compounds partially to replace distillation. This article focuses on selected food products with varying physical properties and chemical composition and will illustrate the general applicability of membrane technology in the food industry.

Dairy Industry

Milk

The dairy industry probably accounts for the largest share of installed membrane capacity among food-processing applications. **Figure 1** is a general schematic of possible applications of membranes in the processing of milk. Reverse osmosis (RO) is mostly used to preconcentrate milk prior to evaporation (although there are RO techniques that could concentrate skim milk up to 45% solids, as with the Freshnote process, described later). This not only saves sufficient energy to justify the technology, but it also